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**MPCR Kit for Human Thrombosis Point
Mutation Detection**
Cat No. MP-70180: 50 reactions
Cat No. MP-70179: 100 reactions

**INSTRUCTION
MANUAL**

ID-M10093
Revised August 9, 2002

*These products are designed and sold for use in the Multiplex PCR (MPCR) covered by patent # 5,582,989. Use of the MPCR process requires a license. A limited, non-automated research field license under the patent to use only this amount of the product to practice the MPCR process is conveyed to the purchaser by the purchase of this product.

The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license. A license for diagnostic purposes may be obtained from Roche Molecular System. A license for research may be obtained by the purchase and the use of authorized reagents and DNA thermocyclers from the Perkin-Elmer Corporation or by negotiating a license with Perkin-Elmer.

This product is intended for research use only and not for diagnostic purposes.

INTRODUCTION

Venous Thromboembolism is a major medical problem, with an annual incidence in the general population of approximately 1 in 1000. An increased risk of venous thrombosis has been found to be associated with several hereditary abnormalities of the anticoagulant pathway involving the Factor V Leiden Mutation (Arg506Gln), eventually leading to activated protein C resistance.

Another candidate gene for venous thrombosis is the prothrombin gene. The mature prothrombin protein is the precursor of the serine protease thrombin, which is an enzyme with procoagulant, anticoagulant, and fibrinolytic activities. The G20210A allele in the 3' untranslated region of the prothrombin gene has been reported to have been associated with the elevated plasma prothrombin levels and increased risk of venous thrombosis.

Maxim has developed a multiplex amplification kit to detect site-directed mutagenesis for one-step determination of Factor V Leiden and G20210A presence in the prothrombin gene. Maxim's human Thrombosis Mutation Point Detection MPCR kits have been designed to detect the expression of human wild type coagulation factor II, mutant coagulation factor II mutant, coagulation factor IX, coagulation factor V wild type, and coagulation factor V mutant. The PCR primers have similar T_m and no obvious 3'-end overlap to enhance multiple amplifications. The 340 bp (wPTH), 340 bp (mPTH), 218 bp (FIX), 180 bp (wFLV), and 180 bp (mFLV) PCR products can be generated from human RNA or the positive control, which is included in this kit.

PCR PRODUCT QUANTITATION

I: Radioactive Quantitation

In our experience, visual inspection of an EtBr-stained agarose gel is sensitive and precise enough to detect changes as low as two-fold. If greater discrimination is necessary, several methods are available. The simplest procedure is to add a radioactively labeled dNTP to the PCR reaction. After gel analysis, the band may be excised and counted in a scintillation counter. Alternatively the gel may be dried and an autoradiogram may be generated which can be scanned in a densitometer. Another method is to label the 5' end of one or both of the primers with ^{32}P , which is incorporated into the PCR products and then assayed for radioactivity (9).

Southern blot hybridization with synthetic DNA probes may also be performed to verify and quantitate PCR generated products, either by densitometry of an autoradiogram or by excising and counting the signal from a hybridization membrane. This method also quantitates only the target product without interference from nontarget products or primer-generated artifacts.

II: Non-Radioactive Quantitation

Nonradioactive quantitation methods include the use of biotinylated or digoxigenin-labeled primers in conjunction with the appropriate detection methods (10), use of a bioanalyzer or WAVE. For an in-depth discussion of the various methods of PCR product quantitation, refer to the review article by Bloch (11).

In addition to the above methods, several companies now offer gel video systems which can scan and quantitate EtBr-stained gel bands in much the same way a densitometer does. Lab-on-a-chip (BioAnalyzer), CE, HPLC, and WAVE may also be used to analyze MPCR products and quantitate simultaneously.

COMPARISON OF MPCR WITH RPA

MPCR (Multiplex Polymerase Chain Reaction)	RPA (RNase Protection Assay)
√ Non-isotope method with high sensitivity 0.1-1 μg total RNA per MPCR	√ Isotope or Non-Isotope methods 1-20 μg total RNA per RPA assay
√ Whole process takes only a few hours	√ Whole process takes two days
√ Detect Multiple Genes Simultaneously & Quantitatively	√ Detect Multiple Genes Simultaneously & Quantitatively
√ Signal can be quantified directly from gel if isotope is included in MPCR. Additional techniques can be used to quantify MPCR product (using Bioanalyzer, HPLC, and WAVE.)	√ Signal can be quantified directly from gel
√ Non-specific products can be eliminated by using probes and southern hybridization.	√ Non-specific signal can be generated by either low stringent conditions or high-secondary-structure template.
√ Ready-to-use	√ Make own "hot" RNA probes

MPCR KIT DESCRIPTION

MPCR Amplification Kits include all necessary MPCR amplification reagents with the exception of *Taq* Polymerase. These kits have been designed to direct the simultaneous amplification of specific regions of human DNA.

MPCR Kits come in two quantities:

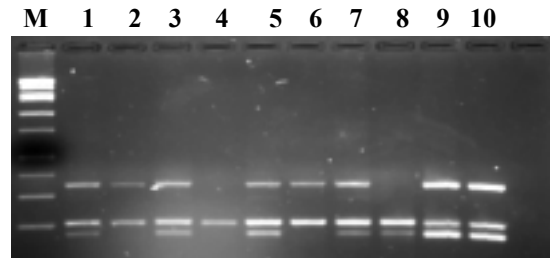
- 50X 50 μ L reaction kits
- 100X 50 μ L reaction kits

Each kit offers Maxim's optimal primer/buffer system which will enhance amplification specificity.

Figure 1 shows quality control MPCR results obtained by following MPCR kit manual using different concentrations of positive control.

For optimal results, please read and follow the instructions in this manual carefully. If you have any questions, please contact Maxim Biotech Customer Service at (650) 871-1919.

Figure 1



Genomic DNA quality control validation of human thrombosis MPCR kit

M: DNA M. W. Marker

Lane 1:PCR using wild type MPCR primers with patient #1 DNA

Lane 2:PCR using mutant type MPCR primers with patient #1 DNA

Lane 3:PCR using wild type MPCR primers with patient #2DNA

Lane 4:PCR using mutant type MPCR primers with patient #2DNA

Lane 5:PCR using wild type MPCR primers with patient #3 DNA

Lane 6:PCR using mutant type MPCR primers with patient #3 DNA

Lane 7:PCR using wild type MPCR primers with patient #4 DNA

Lane 8:PCR using mutant type MPCR primers with patient #4 DNA

Lane 9:PCR using wild type MPCR primers with wild type positive

Lane 10:PCR using mutant type MPCR primers with mutant type positive

MPCR PRIMER INFORMATION

Product Code	Gene	5'/3' Tm	Amplicon Size	Accession No.	Intron Span	Genomic Size
hTHR-wPTH	Human wPTH	57.8°C/60.4°C	341bp	M17262	no	341bp
hTHR-mPTH	Human mPTH	57.8°C/59.9°C	341bp	M17262	no	341bp
hTHR-FIX	Human FIX	54.8°C/51.6°C	219bp	K02402	no	219bp
hTHR-wFVL	Human wFLV	58.1°C/61.7°C	181bp	Z99572	no	181bp
hTHR-mFVL	Human mFLV	58.1°C/59.5°C	181bp	Z99572	no	181bp

KIT COMPONENTS

MP-70180

50X50 μ L MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
hTHR-B001	2X hTHR MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l
hTHR-C001 (Purple)	10X hTHR Pos. Control-Wild Type	50 μ l
hTHR-C002 (Brown)	10X hTHR Pos. Control-Mutant	50 μ l
hTHR-P001 (Yellow)	10X hTHR MPCR Primer-Wild Type	125 μ l
hTHR-P002 (Green)	10X hTHR MPCR Primer-Mutant	125 μ l
MRB-0014	DNA M.W. Marker (100 bp ladder)	100 μ l
MRB-0011P	ddH ₂ O (DNase free)	2.0 ml
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MP-70179

100X50 μ L MPCR reaction kit
Store all reagents at -20°C

Product Code	Kit Component	Amount
hTHR-B001	2X hTHR MPCR Buffer (containing chemicals, enhancer, stabilizer and dNTPs)	1250 μ l x 2
hTHR-C001 (Purple)	10X hTHR Pos. Control-Wild Type	50 μ l x 2
hTHR-C002 (Brown)	10X hTHR Pos. Control-Mutant	50 μ l x 2
hTHR-P001 (Yellow)	10X hTHR MPCR Primer-Wild Type	125 μ l x 2
hTHR-P002 (Green)	10X hTHR MPCR Primer-Mutant	125 μ l x 2
MRB-0014	DNA M.W. Marker (100 bp ladder)	100 μ l x 2
MRB-0011P	ddH ₂ O (DNase free)	2.0 ml x 2
	Instruction Manual	

NOTE: SPIN ALL TUBES BEFORE USING AND VORTEX ALL REAGENTS FOR AT LEAST 15 SECONDS BEFORE USING!!

PROCEDURE

Blood DNA Isolation Protocol

This Blood DNA isolation is adapted from Maxim SA-40007/SA-40008 Blood DNA Spin Column Preparation Kit

- 1. Pipette 300 μ L of BD-1 Solution into a 1.5mL centrifuge tube. Add 300 μ L of whole blood and mix by inverting the tube 3-5 times.**
- 2. Centrifuge for 1 minute at 10,000 x g.**
- 3. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper. Take care that the pellet remains in the tube.**
Note: In rare instances the pellet may be loose, so pour slowly. Inverting the tube on the absorbent paper minimizes chance of the supernatant backflow onto the pellet.
- 4. Add 300 μ L Lysis Buffer. Close the tube and vortex for 20 seconds.**
Note: When processing multiple samples, vortex each tube immediately after the addition of Lysis Buffer.
- 5. Place the tube in a heating block or water bath and incubate at 65°C for 5 minutes.**
- 6. Centrifuge for 1 minute at 10,000 x g.**
- 7. Pipette 120 μ L Isopropanol into a 1.5mL centrifuge tube. Pour the supernatant into the tube without disturbing the pellet and mix by inverting the tube 3-5 times.**
Note: Complete mixing with isopropanol is vital to precipitate DNA. In samples with low white blood cell counts, invert the tube at least 20 times at DNA may not be visible.
- 8. Pour the above mixture into a spin column and centrifuge for 1 minute at 10,000 x g.**
- 9. Add 500 μ L Washing Buffer into the spin column and centrifuge for 1 minute at 10,000 x g.**
- 10. Transfer the spin column into a new centrifuge tube and centrifuge for one minute at 10,000 x g.**
Note: This avoids any residual washing buffer.
- 11. Add 100 μ L Elution Buffer to the spin column and centrifuge for 1 minute at 10,000 x g.**
Note: Eluted DNA is stable if kept at 4°C.

PROCEDURE

1. *Taq* DNA polymerase from Perkin-Elmer or its derivatives are highly recommended for MPCR. Ampli-*Taq* Gold, however, is not recommended because its own optimal buffer system is required.

2. **Reaction Mixture Preparation:**

A. Set up MPCR reactions with the test samples and MPCR buffers provided in the MPCR kit according to the table below:

Volume (Per assay)	Reagent (Add in order)
25.0 µl	2X MPCR BufferMixture
5.0µl	10X MPCR Primers
0.5µl	<i>Taq</i> DNA Polymerase(5U/ml)
5.0µl	Specimen cDNA or
	10X Control cDNA from kit
14.5µl	H ₂ O
50.0µl	Mineral Oil (optional)

B. EDTA concentration in test sample must not exceed 0.5 mM because Mg⁺⁺ concentration in MPCR Buffers is limited to certain ranges. Additional Mg⁺⁺ may be added to the PCR mixture to compensate for EDTA. We strongly recommend running an MPCR reaction with the positive control provided in the kit. Since the MPCR DNA polymerase needed in each reaction is in a very small volume, it is recommended that all of the PCR components be premixed in a sufficient quantity for daily needs and then dispensed into individual reaction vials. This will help you to achieve more accurate measurements.

3. **PCR thermocycle profile:**

Reaction profiles will need to be optimized according to the machine type and needs of user. Please take note that temperature variations occur between different thermocyclers, therefore, the annealing temperature in the sample profile below is given as a range. It will be necessary to determine the optimal temperature for your individual thermocycler. An example of a time-temperature profile for the positive control PCR reaction optimized for Perkin Elmer machine types 480, 2400, and 9600 is provided below:

Temperature	Time	Cycles
96°C	1 min	2X
58-60°C	4 min	
94°C	1 min	28-35X
58-60°C	2 min	
70°C	10 min	1X
25°C	soak	

Note: A 2-step PCR thermocycle profile was found to be more effective than a 3-step PCR thermocycle profile for MPCR amplification. For 2-step PCR, use 94-95°C for denaturation and 58-60°C for annealing and extension. The 72°C step is omitted.

4. **Agarose Gel Electrophoresis:**

To fractionate the MPCR DNA product electrophoretically, mix 10µl of the MPCR product with 2µl 6X loading buffer. Run the total 12µl alongside 10 µl of DNA marker* from the MPCR kit on a 2 % agarose gel containing 0.5 mg/ml ethidium bromide. Electrophorese and photograph. (Hint: Best results are obtained when the gels are run slowly at less than 100 volts).

* DAN Marker contains linear double stranded DNA bands of 1,000; 900, 800, 700; 600; 500; 400; 300; 200; and 100 base pairs (bp).

TROUBLESHOOTING

1. MPCR AMPLIFICATION

Observation	Possible Cause	Recommended Action
1.1. No signal or missing some bands during amplification even using positive control provided in kit.	1.1a. The annealing temperature in the thermocycler is too high. 1.1b. Dominant primer dimers.	1.1a. Decrease PCR annealing temperature 3-5°C gradually. 1.1b. Use any one of "Hot Start" PCR procedures.
1.2. Too many nonspecific bands.	1.2a. The annealing temperature in the thermocycler is too low. 1.2b. Pre-PCR mispriming. 1.2c. cDNA is interfering with MPCR	1.2a. Increase PCR annealing temperature 3-5°C gradually. 1.2b. Use any one of "Hot Start" PCR procedures. 1.2c. Clean cDNA with Phenol/ Chloroform. 1.2d. Use Maxim's 3M™-MPCR Kit.
1.3. No difference in gene expression among treatments	1.3a. PCR amplification of this specific gene has passed the exponential phase. 1.3b. Variation in sample preparation, RT reaction and amounts of input cDNA.	1.3a. Decrease PCR cycle number or decrease the input cDNA. 1.3b. Run a parallel PCR with a house-keeping gene to eliminate variables.

PRECAUTIONS AND STORAGE

Storage

1. Store all MPCR Kit components at -20°C. Under these conditions components of the kit are stable for 1 year.
2. Isolate the kits from any sources of contaminating DNA, especially amplified PCR product.
3. Do not mix MPCR kit components that are from different lots. Each lot is optimized individually.

REFERENCES

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